REPORT

Roles of antimicrobial peptides such as defensins in innate and adaptive immunity

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A number of antimicrobial peptides such as defensins have multiple functions in host defence. Defensins are produced not only by phagocytic cells and lymphocytes, but also by the epithelial cell lining of the gastrointestinal and genitourinary tracts, the tracheobronchial tree, and keratinocytes. Some are produced constitutively, whereas others are induced by proinflammatory cytokines and exogenous microbial products. Defensins produced by cells in the course of innate host defence serve as signals which initiate, mobilise, and amplify adaptive immune host defences. Administration of defensins with antigens to mice enhances both cellular (Th1-dependent) and humoral (Th2-dependent) cytokine production and immune responses. Linkage of defensins to weak tumour antigens potentiates their immunoadjuvant effects. Defensins use multiple cellular receptors, which endows them with the capacity to marshall adaptive host defences against microbial invaders.

Animals live in an environment filled with pathogenic microorganisms. If the epithelial barrier is breached by an injury, pathogens that invade the host are eliminated initially by innate, followed by adaptive, host immune reactions. Innate immunity consists of a range of pre-existing rapidly mobilised host defences including neutrophil and macrophage phagocytic cells, epithelial cells, mast cells, eosinophils, and natural killer cells.[1 2] These cells express a wide variety of pattern recognition receptors, such as toll-like receptors (TLRs), C lectin receptors, and scavenger receptors, which are activated by components of microbial pathogens. This results in the release and/or activation of numerous effector molecules and mediators of host defence including the complement cascade, cytokines, chemokines, superoxides, nitric oxides, prostaglandins, acute phase proteins, and antimicrobial peptides. In this paper, we will review the multiple means by which antimicrobial peptides—defensins and cathelicidin in particular—activate not only innate but also adaptive immune responses.

Adaptive immune responses are activated by the following sequence of cellular interactions. Specialised cellular members of the innate immune system function to activate adaptive immune reactions. They consist of pre-existing Langerhans cells of the skin and immature dendritic cells (iDCs), which can phagocytose microbial proteins and process them into small peptide fragments.[3] As the dendritic cells mature in response to inflammatory stimuli, these small (8–9 amino acid) peptides are exported on to the cell surface together with MHC proteins.[3] The complex of MHC and antigenic epitopes is presented by these now mature dendritic cells to those T cells in the lymph nodes that express a complementary T cell receptor for the antigen. Class I MHC-antigen complexes preferentially activate CD8 cytotoxic T cells, whereas class II MHC-antigen complexes activate CD4 helper T cells, which produce immunoenhancing cytokines that promote adaptive B cell proliferation and antibody production. Other cytokines produced by T cells in turn activate phagocytic macrophages and neutrophils as well as natural killer cells, resulting in positive feedback augmentation of innate defences.

A wide variety of host proteins have been shown to have antimicrobial activity. Antimicrobial peptides such as defensins are widely distributed in nature and are found not only in vertebrates, but also in invertebrates and even in the plant kingdom.[4] Antimicrobial peptides are produced by leucocytes and epithelial cells lining the environmental interface of the gastrointestinal and genitourinary tracts, tracheobronchial tree, and skin. They include defensins, cathelicidin, histatins, cathepsin G, azurocidin, chymase, eosinophil derived neurotoxin, high mobility group 1 nuclear proteins, HMGB1, lactoferrin, and many others.[4–8] This overview will focus on defensins and cathelicidins of mice and men.

Many of the antimicrobial peptides are constitutively present in cells and stored in secretory granules. Others are induced by proinflammatory stimuli. They exhibit large cationic patches on their molecular surface which enables them to depolarise and/or pierce bacterial cell membranes. However, the antibacterial and antifungal activities of defensins, cathelicidins, and selected chemokines are inhibited by physiological salt concentrations and by serum.[5 6] Consequently, their microbicidal activities may be exerted largely in phagocytic vacuoles and on the external surface of the skin and mucosa.[7 8] Some of the antimicrobial peptides have potent in vitro antiviral activities. Both α defensins and eosinophil derived neurotoxin have been reported to have considerable anti-HIV-1 activity.[9 10] In fact, α defensins are said to account for the soluble CD8 antiviral factor secreted by CD8 T lymphocytes from long term non-progressing HIV-1 positive patients.[9]

STRUCTURE OF DEFENSINS AND CATHELICIDIN

The defensins can be classified into two subfamilies based on their tertiary structure. They exhibit considerable variation in their amino acid sequences, perhaps based on selective pressures to enable them to contend with a wide variety of microbial agents. The human α defensins, although small (3.5–4 kDa), have three intramolecular cysteine bonds linking cysteines 1–6, 2–4, and 3–5, whereas the β defensins (4–6 kDa) have bonds between cysteines 1–5, 2–4, and 3–6. Consequently, these small molecules have an intricate tertiary structure with a core of three anti-parallel β sheet components resembling chemokines.[11 12]

In contrast, cathelicidin (LL37) consists of the linear C terminus of the human CAP-18 molecule, has an α-helical structure, and interacts with the formyl peptide receptor-like 1 (FPRL1) G protein coupled receptor rather than with a chemokine receptor.[13 14]

Abbreviations: iDC, immature dendritic cell; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MBD, murine β defensin; TLR, toll-like receptor; TNF, tumour necrosis factor

Table 1 Cell sources of human defensins and cathelicidin

Peptide	Constitutive expression	Induced by proinflammatory cytokines and endotoxin (LPS)
α Defensins (HNP1–3)	Neutrophil granules	Monocytes[15] CD8 T lymphocytes (CTL)[9–15]
α Defensins (HD5–6)	Paneth cell granules	
β Defensins (HBD1)	Keratinocytes Barrier epithelial cells	Keratinocytes[35] Monocytes and dendritic cells[36]
β Defensins (HBD2–4)		Monocytes and dendritic cells[36] Keratinocytes[14 19] Barrier epithelial cells[14 19] Mast cells[37]
Cathelicidin (LL-37)	Neutrophil granules	Keratinocytes[13 15] Barrier epithelial cells[13 15] Monocytes[15] T lymphocytes[15] Mast cells[38]

LPS, Lipopolysaccharide.

CELL SOURCES AND INDUCTION OF DEFENSINS AND CATHELICIDIN

The α defensins, also known as the human neutrophil peptides (HNP1–3), are largely stored in the granules of neutrophils and to a lesser extent in macrophages (table 1). They are constitutively produced by myeloid precursor cells. However, recent reports suggest that their production can be induced by activated CD8 T cells as well.[9 15] In contrast, HD5 and HD6 are stored in the granules of Paneth cells at the base of villi in the gastrointestinal tract. When induced to degranulate, neutrophils and Paneth cells release these α defensins locally. The pathophysiological roles of the various defensins have been difficult to establish because there are too many genes to readily delete. However, knockout mice lacking the matrilysin (metalloproteinase 7) enzyme, which is required to cleave the proforms of Paneth cell derived HD5 and HD6, did have reduced resistance to gastrointestinal pathogens.[16] As this cleavage is not the only function of matrilysin, this observation is not definitive. There is abundant evidence that α defensin concentrations do become considerably elevated at sites of acute inflammation,[17] and plasma concentrations are increased in systemic infectious diseases.[18]

There are also many β defensins. HBD1 is expressed constitutively by keratinocytes and found in the interstices between the cells. HBD2–4 on the other hand are inducible and produced by keratinocytes and epithelial cells in response to proinflammatory stimuli such as interleukin (IL)-1, tumour necrosis factor (TNF), and lipopolysaccharide (LPS).[14 19] Evaluation of the human genome suggests that there may be an additional 25 β defensins that have not yet been identified.[20] Despite this apparent redundancy, deletion of the gene for HBD1 does delay the capacity of knockout mice to clear haemophillus influenza from the lung[21] and results in increased colonisation by *Staphylococcus* in the bladder.[22] Cathelicidin is both stored in neutrophil granules and is an inducible product of epithelial cells, T cells, and monocytes. Deletion of the murine homologue, CRAMP, reduces resistance of mice to infectious challenge of the skin.[23]

CHEMOTACTIC ACTIVITIES OF DEFENSINS AND CATHELICIDIN

In addition to their innate antibacterial role, many of these peptides have recently been shown to interact with various receptors on iDCs and lymphocytes, resulting in activation of adaptive immune responses. This conclusion is based on the chemotactic effect of antimicrobial peptides for selected leucocytes. This chemotactic ability of the antimicrobial peptides indicates that they can attract host cells expressing the appropriate receptors along a gradient to their site of origin.[24] Furthermore, as their chemotactic responses are inhibitable by pertussis toxin, the antimicrobial peptides interact with Gαi protein coupled receptors. The receptor has been identified in the case of β defensins as CCR6, which is shared by the chemokine MIP3α/LARC/CCL20.[25] The α defensins were reported to interact with the G protein coupled receptor for adrenocorticotrophic hormone a number of years ago, which may account for their capacity to suppress glucocorticoid production.[26] This suppressive effect would be predicted to further promote the immunoenhancing capabilities of α defensins. Although the chemotactic effects of cathelicidin for neutrophils, monocytes, and T lymphocytes are also pertussis toxin inhibitable, it does not interact with a chemokine receptor, but with FPRL1.[13]

Predictably, human and murine defensins are chemotactic for those cells that express the appropriate receptors (table 2). Although the receptor for α defensins has not been identified as yet, the chemotactic effects of α defensins are inhibitable by pertussis toxin, identifying it as a Gαi protein coupled receptor. The α defensins are chemotactic for immature, but not mature, dendritic cells and the subset of naive resting CD4CD45RA and some CD8 T lymphocytes, but not neutrophils or monocytes.[27] The β defensins are selectively chemotactic only for CCR6 expressing cells, including iDCs and resting memory CD4CD45RO as well as some CD8 T lymphocytes.[25] HBD3 is also chemotactic for monocytes, which do not express CCR6, suggesting it uses an additional chemotactic receptor.[28] The defensins do not induce calcium flux. On

Table 2 Chemotactic targets of human defensins and cathelicidin

Peptides	Monocytes	Endothelial cells	Neutrophils	Mast cells	T Cells	iDCs
α Defensins 1–3	–	+[18]	–	ND*	Naive + CD8[27]	+[27]
β Defensins 1–2	–	ND	–	–[30]	Memory + CD8[25]	+[27]
β Defensins 3–4	+[28]	ND	–	ND	Memory + CD8[25]	+[25]
Cathelicidin (LL37)	+[13]	ND	+[13]	+[31]	+[13]	–

iDC, Immature dendritic cell; ND, not determined.

Table 3 Activities of human defensins

Peptide	Innate and adaptive immune effects
α Defensins (HNP1–3)	Antimicrobial and antiviral (anti-HIV-1)[4–8 10 39] Regulate complement activation[40 41] Degranulate mast cells[42] Induce pulmonary epithelial cell proliferation in vitro[43] Inhibit glucocorticoid production by blocking ACTH receptor[26] Block LPS binding to LBP[44] Chemotactic for naive resting T cells, CD8 T cells, immature dendritic cells[27] Immunoadjuvant effects in mice[30 31] Augment cytokine production (IL5, IL6, IL10, and IFNγ) Promote antigen induced ex vivo splenocyte proliferation Enhance antigen induced cellular and humoral immune responses
β Defensins (HBD1–3)	Antimicrobial[4–8] Induce prostaglandin D_2 production[29] Degranulate mast cells[29] Chemotactic for CCR6 dendritic cells[25] HBD3 also acts on unknown GPCR on monocytes[15] Immunoadjuvant effects in mice[32 33] Enhance tumour antigen induced humoral and cellular immunity HBD2 induces cytokines and chemokines HBD2 also activates TLR4 iDCs

ACTH, Adrenocorticotrophic hormone; GPCR, G protein coupled receptor; iDC, immature dendritic cell; IFN, interferon; IL, interleukin; LPB, lipopolysaccharide binding protein; LPS, lipopolysaccharide; TLR, toll-like receptor; TNF, tumour necrosis factor.

the basis of usage of chemokine receptors and the tertiary structural similarities between defensins and chemokines,[12] the defensins can actually be considered to be "microchemokines" which act on cells of the adaptive immune system.

BIOLOGICAL ACTIVITIES OF DEFENSINS AND CATHELICIDIN

The α defensins have been reported to have a number of biological activities (table 3). They degranulate mast cells resulting in the release of histamine.[29] Intranasal administration of α defensins along with an antigen enhances the systemic cellular and humoral immune responses to that antigen.[30] This is associated with enhanced ex vivo production of both Th1 and Th2 type cytokines such as IL1, TNF, IL6, IL4, and interferon (IFN)γ by splenocytes from such mice. Systemic injections of human α defensins into mice also results in augmentation of both Th1 and Th2 immune responses to potent as well as weaker tumour antigens.[31] Thus human α defensins are active across species in mice and have potent immunoadjuvant effects.

DEFENSINS AUGMENT IMMUNITY TO TUMOUR ANTIGEN

Although human β defensins are inactive in mice, murine β defensins (MBDs) are chemotactic for subsets of murine T cells and iDCs that express CCR6.[32 33] Furthermore, they also have potent immunoadjuvant activity and promote antitumour adaptive immune responses[34] to injection of mice with plasmids containing cDNA for selected chemokines or β defensins linked to HIV ENV antigen or to a tumour antigen, such as the Fv portion of immunoglobulin expressed by murine B cell lymphomas. Fusion products consisting of chemokines linked to HIV-gp120 will induce mucosal as well as systemic immunity.[34] Although the immunoglobulin "self" antigens were not immunogenic when administered by themselves, they induced murine immune responses when administered intracutaneously using a gene gun or as proteins linked to a β defensin ligand. As unlinked mixtures of the tumour antigen and β defensin or antigen linked to a prodefensin molecule that cannot interact with CCR6 are inactive, it is reasonable to propose that the β defensins facilitated the delivery of antigen to receptors on iDCs. The fact that this approach breaks tolerance to self suggests that delivery of antigens to these receptors favours the processing by iDCs and subsequent immunogenic presentation of the antigen by mature dendritic cells. MBD3 linked to the Fv portion of an immunoglobulin from murine lymphomas when injected into mice induces a considerable antibody response, but does not protect mice against a tumour challenge. On the other hand, MBD2 linked to the same Fv antigen induces moderate levels of antibody to the tumour antigen, but considerable cellular immunity, as 50% of the immunised mice are protected against tumour challenge.[32] Tumour immunity usually involves the participation of Th1 cytokines such as IFNγ and the induction of cytotoxic T lymphocytes. This was also true of the MBD2 induced tumour immunity, because immunisation with MBD2 fused to the tumour Fv antigen failed to protect IFNγ knockout mice. In contrast, the MBD3 fusion product did not induce IFNγ. These perplexing differences in the effects of MBD2 and MBD3, which use the same CCR6 receptors, were therefore further investigated.

MBD2 MATURATION OF DENDRITIC CELLS IS TLR4 DEPENDENT

The possibility that the β defensins had activating as well as chemotactic effects on iDCs was evaluated. Incubation of iDCs with MBD2 or LPS, but not MBD3, induced the differentiation into mature cells which expressed CD11c, CD40, and B7.2 cell surface molecules.[33] These mature dendritic cells became capable of presenting antigen, as evidenced by the fact that they were able to augment a mixed leucocyte (proliferative) response. Furthermore, MBD2, like LPS, induced dendritic cells to produce cytokines, such as IL1, TNF, IL6, and IL12, and chemokines, such as IL8, MDC, IP-10, MIP1-α and to up regulate the expression of the CCR7 receptor (table 4). However, as predicted, maturation of dendritic cells was associated with a decrease in the expression of chemokine receptors such as CCR2, CCR5, and CCR6 and the mannose and scavenger 2 receptors. The possibility that LPS contamination of the recombinant protein accounted for its activating effect on iDCs was ruled out (table 4), as limulus assays failed to detect any LPS in the MBD2 preparation. The activity of MBD2, unlike that of LPS, was destroyed by digestion with proteinase K and boiling for 15 minutes. In contrast, the activity of LPS, but not of MBD2, was blocked by polymixin B, diphosphoryl lipid A from *Rhodobacterium*, and by incubating

Table 4 Comparison of effects on immature dendritic cells (iDCs) of murine BD2 and lipopolysaccharide (LPS)

	LPS	MBD2
Induces iDC chemokine production - IL8, RANTES, MDC, IP10 and MIP1α	+	+
Induces iDC chemokine receptor - CCR7	+	+
Induces iDC cytokine production - IL1α, β, TNF, IL6, IL12, IFNγ	+	+
Suppresses chemokine receptor expression - CCR2, CCR5, CCR6	+	+
Suppresses receptor expression - mannose and scavenger R2	+	+
Blocked by proteinase K and by boiling for 15 min	No	Yes
Blocked by polymixin B and diphosphoryl lipid A	Yes	No
Activates iDCs in serum-free medium	No	Yes
Activates iDCs from CCR6 $^{-/-}$ mice	Yes	Yes
Activates iDCs from TLR4 mutant mice	No	No

IFN, interferon; IL, interleukin; TLR, toll-like receptor; TNF, tumour necrosis factor.

iDCs in serum-free medium lacking LBP for one hour. The activating effect of LPS and MBD2 on iDCs from CCR6 knock-out mice was also compared. Unexpectedly, the MBD2, as well as LPS, was able to induce maturation of iDCs in the absence of CCR6, suggesting that another receptor interaction may be responsible for this effect. Because of the similarity of the effects of LPS and MBD2, their capacity to stimulate iDCs from mice with mutant TLR4 genes was then compared. iDCs obtained from defective C3H/HeJ and C57BL10ScNcr mice with TLR4 gene defects were unresponsive to both LPS and MBD2. MBD2 also induced receptor gene expression of HEK293 cells transiently cotransfected with murine TLR4 and MD2 genes. These results suggested that MBD2 uses TLR4 to induce Th1 immune responses independent of its CCR6 dependent chemotactic effect. Consequently, these data identify MBD2 as a unique endogenous ligand for TLR4 and appear to reflect an additional adaptation by which this defensin molecule is able to activate adaptive immune responses.

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